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Ninety-Day Inhalation Study in Rats, Using Aged and Diluted Sidestream Smoke from a Reference Cigarette: DNA Adducts and Alveolar Macrophage Cytogenetics

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To study the genotoxic effects of subchronic exposure to environmental tobacco smoke, Sprague–Dawley rats were exposed to 0, 0.1, 1.0, and 10 mg total particulate matter (TPM)/m^3 of aged and diluted sidestream smoke (ADSS) from 1R4F reference cigarettes 6 hr per day, 5 days a week for 13 weeks. DNA from lung, heart, larynx, bladder, and liver was tested for adduct formation by the ^{32}P-postlabeling assay after 28 (except bladder) and 90 days of exposure and 90 days after cessation of exposure. In addition, alveolar macrophages from animals exposed for 28 or 90 days were examined for chromosomal aberrations. Exposure-related DNA adducts were not observed in any tissue in any of the animals exposed to 0.1 or 1.0 mg TPM/m^3. However, increased levels of DNA adducts with diagonal radioactive zones were observed in lung, heart, and larynx DNA of animals exposed to the highest concentration of ADSS (10 mg TPM/m^3). Adduct analyses with varying amounts of DNA from lungs of mid- and high-exposure animals clearly indicate that the dose–response for DNA adduct formation is nonlinear. The adduct levels were highest after 90 days of exposure and were significantly reduced in all target tissues 90 days after cessation of exposure. Chromosomal aberrations in alveolar macrophages were not elevated in any group after 28 or 90 days of exposure. These results indicate a no-observed-effect-level (NOEL) of at least 1.0 mg/m^3 for DNA adduct formation in lung, heart, and larynx, and a NOEL of at least 10 mg/m^3 for the induction of chromosome aberrations in alveolar macrophages, under the conditions of this study.

The potential health consequences of exposure to environmental tobacco smoke (ETS) are complex issues which concern both scientists and the general public (Department of Health and Human Services, 1986; National Research Council, 1986). Epidemiological studies intended to address the effects of exposure to ETS have reported both positive and negative associations between lung cancer and exposure to ETS (Department of Health and Human Services, 1986; Layard, 1986). Several *in vitro* studies conducted on highly concentrated extracts of ETS have reported positive results, indicating that it is genotoxic under these study conditions (Claxton *et al.*, 1989; Lewtas *et al.*, 1987; Ling *et al.*, 1987; Lofroth *et al.*, 1988; Lofroth and Lazardis, 1986). However, the results of these *in vitro* studies are of limited value for the evaluation of the effects of exposure to ambient concentrations of ETS. Genotoxic effects of exposure to ETS can be best determined by controlled experiments in which animals are exposed to known concentrations of ETS followed by measurement of sensitive biological markers for genotoxic damage.

ETS consists of aged and diluted sidestream smoke (ADSS, 85%) mixed with aged and diluted exhaled mainstream smoke (15%). Studies (Guerin *et al.*, 1992; Turner *et al.*, 1992) have shown that the concentration of respirable suspended particles (RSP) in offices and restaurants where smoking is unrestricted is approximately 0.1 mg/m^3, rarely exceeding a concentration of 0.3 mg/m^3. Less than 50% of the RSP originated from cigarette smoking (Guerin *et al.*, 1992). However, studies on the biological responses to cigarette smoke exposure at or near these concentrations are scarce (von Myerinck *et al.*, 1989). In our previous study (Lee *et al.*, 1992), rats were exposed to ADSS as a surrogate for ETS for 14 consecutive days. No increase in DNA adducts was observed in animals exposed to ambient (0.1 mg/m^3) and 10-fold exaggerations (1 mg/m^3) of ETS. Adducts were increased in lung and heart but only at 10 mg/m^3, the 100-fold exaggerated level of the ambient ETS concentration. Cytogenetic analysis in pulmonary alveolar macrophages was completely negative at all ADSS concentrations (Lee *et al.*, 1992).

In the present study, we have significantly extended both the length of the ADSS exposure and the length of the post-exposure recovery period. Rats were exposed to ADSS at

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concentrations of 0.1, 1.0, and 10 mg/m^3 for 90 days followed by 90 days of recovery. Two sensitive biological markers for genotoxicity, chromosomal aberrations in pulmonary alveolar macrophages (PAM) and DNA adduct formation in major target and nontarget organs, were analyzed after 28 and 90 days and after the recovery period. The results of the 90-day study confirm the existence of a no-observed-effect-level (NOEL) of at least 1.0 mg/m^3 for DNA adduct formation and a NOEL of at least 10 mg/m^3 for the induction of chromosomal aberration in PAMs even in the extended exposure duration of 90 days.

MATERIALS AND METHODS

Materials. Calf spleen phosphodiesterase (2 U/mg) was obtained from Boehringer-Mannheim (Indianapolis, IN). Micrococcal nuclease (100–200 U/mg) and nuclease P1 (255 U/mg) were from Sigma (St. Louis, MO). T_4 polynucleotide kinase was from GIBCO–BRL (Gaithersburg, MD), and [γ-^{32}P]ATP (3000 Ci/mmol) was from New England Nuclear (Wilmington, DE). Polyethyleneimine cellulose (PEI-cellulose) coated thin-layer chromatographic (TLC) sheets (Machery Nagel) were purchased from Brinkmann Scientific (Westbury, NY). The 1R4F reference cigarettes (0.8 mg nicotine and 11.5 mg tar per cigarette) were obtained from the Tobacco and Health Research Institute, Lexington, KY.

Experimental animals. Details on experimental animals have been reported elsewhere (Coggins *et al.*, 1993). Briefly, 5-week-old male Sprague–Dawley rats (Charles River, Raleigh, NC) were acclimated for 2 weeks prior to exposure in 2-M^3 stainless steel inhalation chambers. The animal rooms had controlled lighting (12 hr dark and 12 hr light), temperature (20–24°C), and humidity (40–60% relative humidity). Animals were allowed unrestricted access to feed (Purina Rodent Chow 5002) and distilled water, except during the smoke exposures. The animals were housed and cared for in accordance with the Animal Welfare Act of 1970 and amendments (Public Law 91-579), as set forth in CFR Title 9, Part 3 Subpart F.

Smoke generation and exposure conditions. Smoke generation and exposure conditions have been described previously (Coggins *et al.*, 1993). Briefly, smoke was generated from 1R4F research cigarettes with a 30-port AMESA generator (CH Technologies, Westwood, NJ) fitted with an aluminum cone for collection of sidestream smoke. Cigarettes were smoked according to the Federal Trade Commission method (a 35-ml puff of 2 sec duration, once per minute) except that instead of a fixed butt length, a fixed number of puffs (7) was taken (Baumgartner and Coggins, 1980; Ayres *et al.*, 1990). Mainstream smoke was exhausted and discarded and the sidestream smoke was drawn into a common plenum. Different amounts of aged and diluted sidestream smoke were provided for each chamber and mixed with dilution air drawn from the animal room through HEPA filters. Target concentrations for suspended particulates were 0, 0.1, 1, and 10 mg/m^3. Exposures were for 6 hr per day, 5 days a week for 13 weeks (total of 65 exposures). Dermal absorption and oral ingestion of particulates after preening were minimized by the use of nose-only restraint tubes inside the chambers.

Experimental design. Rats were divided into four exposure groups: sham, low (0.1 mg TPM/m^3), medium (1.0 mg TPM/m^3), and high (10.0 mg TPM/m^3) exposures. For DNA adduct analysis, each group contained 15 male animals. Five animals in each group were killed after 28 and 90 days of exposure for DNA adduct analysis. The remaining 5 animals in each group were kept for a further 90 days without smoke exposure for the reversibility study. For chromosomal aberrations in PAM, 5 animals in each exposure group were killed after 28 and 90 days of exposure. No reversibility study was conducted for chromosomal aberrations in PAM.

DNA isolation. DNA was isolated and purified as described previously (Lee *et al.*, 1992). Briefly, whole lung, heart, liver, larynx, and bladder samples were homogenized, centrifuged, and the supernatant extracts discarded. Pellets were resuspended and DNA was isolated on a Genepure 341 nucleic acid purification system (Applied Biosystem, Inc., Foster City, CA) by solvent extraction and enzymatic digestion of protein and RNA. The DNA concentration was estimated spectrophotometrically prior to analysis ($1 A_{260} = 50$ μg DNA/ml). Absorbance ratios (260/280) of all DNA samples ranged from 1.6 to 1.8.

^{32}P-postlabeling assay. Five micrograms of DNA was digested to 3′-nucleotides by incubation at 37°C for 3.5 hr in a total volume of 10 μl containing 0.6 U of micrococcal nuclease and 5.0 μg of spleen phosphodiesterase. Adducted nucleotides were enriched by the nuclease P1 procedure (Reddy and Randerath, 1986) or by butanol extraction (Gupta, 1985) and samples were then [5′-^{32}P]phosphorylated at 37°C using 75 μCi of [γ-^{32}P]ATP with a specific activity of ~3000 Ci/mmol and 4.5 U of T_4 polynucleotide kinase (Lee *et al.*, 1992). ATP excess was confirmed by one-dimensional separation of normal nucleotides from unreacted [^{32}P]ATP in PEI-cellulose TLC using 0.28 M ammonium phosphate/0.05 M sodium phosphate, pH 6.0. Resolution of the ^{32}P-labeled adducts was carried out as previously described (Gupta *et al.*, 1982) with 4 μg of DNA applied on PEI-cellulose TLC sheets using the following solvents: D1, 1.0 M sodium phosphate, pH 6.0 (overnight onto a wick); D3, 5.3 M lithium formate, 8.5 M urea, pH 3.5; D4, 1.2 M lithium chloride, 8.5 M urea, 0.5 M Tris base, pH 8.0 (onto a wick); D5, 1.7 M sodium phosphate, pH 6.0 (overnight onto a wick).

Quantification of DNA adducts. The specific activity of each batch of [^{32}P]ATP was determined by measuring the kinase-catalyzed incorporation of radioactivity into 10 pmol of 2′-deoxyadenosine 3′-monophosphate (dAp) (Reddy and Randerath, 1986). Values of specific activity fell within the range of 1.5 to 2.5×10^6 cpm/pmol. TLC plates were wrapped in Mylar plastic and scanned for 8 hr using the AMBIS radioanalytic imaging system (AMBIS Systems, Inc., San Diego, CA) under an argon atmosphere. The counting efficiency averaged approximately 20%; a 3.2 × 3.2-mm resolution plate was used. The use of the AMBIS radioanalytic imaging system in two-dimensional TLC and in ^{32}P-postlabeling assay of DNA adducts has been previously reported (Hook *et al.*, 1990; Turteltaub *et al.*, 1990; Lee *et al.*, 1992). The adduct maps comparing the conventional autoradiography and AMBIS scanning are shown in Fig. 1. Relative adduct labeling (RAL) values were calculated by

$$\text{RAL} = \frac{\text{cpm in adduct nucleotides}}{\text{sp act (ATP)} \times \text{pmol dNp applied to TLC}}$$

In vitro modification of DNA with cigarette smoke condensate (CSC). In order to prepare the DNA–CSC adduct to be used as a positive control, 1 mg of lung DNA isolated from Sprague–Dawley rats was treated with 5 mg (in 500 μl of DMSO) of CSC from Kentucky Reference 1R4F cigarettes in the presence of an S9 metabolic activation system (Ames *et al.*, 1975). The S9 concentration was 5% (v/v) and 5.0 ml of the S9 mix was added to the reaction tube. The reaction mixture was incubated for 5 hr at 37°C. DNA was extracted with the ABI Genepure Extractor.

In vivo modification of DNA with benzo[a]pyrene (B[a]P). Female B6C3F1 mice were given a single ip dose of 40 or 60 mg/kg of B[a]P in 0.1 ml of DMSO/corn oil (50/50, v/v). Livers were collected 43 hr after B[a]P administration. DNA was extracted by the Genepure Extractor and used as a source of standard DNA–B[a]P adducts.

Pulmonary alveolar macrophage isolation and analysis of chromosomal aberration. Designated animals were injected ip with colchicine (6 mg/kg, 12 mg colchicine/ml Hank's balanced salt solution (HBSS) stock solution) at the end of the 28- and 90-day time points in the study. Four hours later animals were killed by asphyxiation with 70% carbon dioxide (CO_2) and the tracheas were cannulated with 14G iv catheters. The lungs and

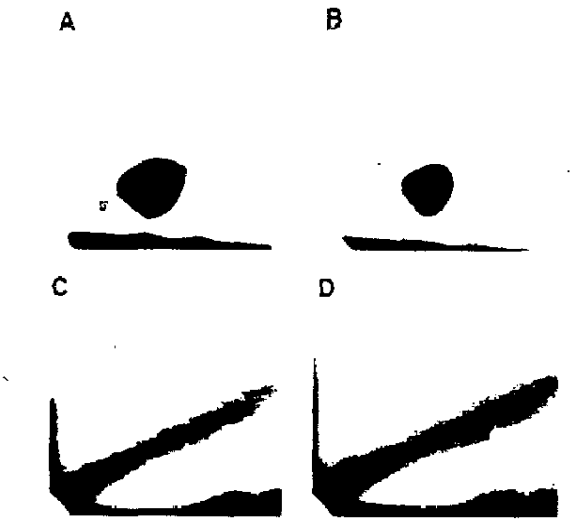

FIG. 1. TLC maps of B(a)P-DNA and CSC-DNA adducts developed by AMBIS scanning and autoradiography. B(a)P-DNA adducts (A, B) were prepared from liver of B6C3F1 mouse injected ip with 40 mg/kg of B(a)P and assayed by P1 nuclease enhancement. CSC-DNA adducts (C, D) were prepared *in vitro* as described under Materials and Methods. The adduct assays were done with 1.6 μg of B(a)P-modified DNA and 4 μg of CSC-modified DNA. TLCs were scanned 2 and 10 hr with AMBIS radioanalytic imaging system for B(a)P-DNA (A) and CSC-DNA (C), respectively. For autoradiography, Kodak XAR-5 diagnostic films were exposed for 7 and 24 hr for B(a)P-DNA (B) and CSC-DNA (D), respectively, at −80°C.

heart were removed and placed in ice-cold HBSS (Ca^{2+} and Mg^{2+} free) for 30 min. The lungs were lavaged and massaged repeatedly using a 10-ml syringe filled with 7 ml of ice-cold HBSS. The lavage fluid was collected in a 50-ml centrifuge tube on ice. The lavage fluid was centrifuged and the supernatant fluid drawn off. The pellet was resuspended in prewarmed (37°C) 75 mM KCl and incubated at 37°C for 25 min. One milliliter of modified Carnoy's (6:1 absolute methanol/glacial acetic acid, v/v) was added to the tubes before centrifugation. After centrifugation the cells were fixed first with modified Carnoy's and then three more times with Carnoy's fixative (3:1). Metaphase spreads were prepared. Slides were then stained for 5 min with Giemsa diluted to 1:20. Fifty metaphases for each animal were scored for chromosome aberrations. Scoring was done without knowledge of treatment group.

Statistical analysis. For DNA adduct data, Levene's test for equality of variance was conducted. Since unequal variances were found, data were log-transformed. Statistical significance ($p < 0.05$) was evaluated by analysis of variance followed by pairwise student's t tests with Bonferroni adjustment. Fisher's exact test was used to determine the level of statistical significance for chromosomal aberration data.

RESULTS

Inhalation of the ADSS by the rats was confirmed by measurement of blood carboxyhemoglobin and plasma nicotine/cotinine (Coggins *et al.*, 1993). No overt signs of toxicity were observed and no significant body weight differences were noted between any of the exposed groups and the sham controls. All lungs, including the ones from the high-exposure group, appeared normal at necropsy. The only histopathological changes observed were mild hyperplasia of the epithelium of the nasoturbinates, in one section of the nose, in the high-exposure group only. These histopathological changes did not progress with increased exposure and they were reversed after cessation of ADSS exposure (Coggins *et al.*, 1992, 1993).

After 28 days of exposure, adduct levels in all the organs tested (lung, heart, larynx, liver) in animals from low- and medium-exposure groups were similar to those of sham control animals (adduct maps not shown). A few discrete adduct spots, thought to represent endogenous DNA adducts, were visible in both groups and the sham controls. Significantly elevated levels of DNA adducts (Fig. 4) were observed in lung, heart, and larynx DNA of animals from the high-exposure group (10 mg/m^3). ^{32}P maps (not shown) of these DNA samples exhibited a few discrete adduct spots, more intense than found in the sham and low- and medium-exposure groups, and diffuse diagonal radioactive zones (DRZ) extending from close to the origin to the middle right-hand margin of the TLC sheet. The DRZ, presumably consisting of numerous incompletely resolved DNA adducts, appears to be characteristic of DNA adducts observed after tobacco smoke exposure (Randerath *et al.*, 1988). DRZs were observed in the adduct maps of CSC–DNA adduct prepared *in vitro* but not in the benzo[*a*]pyrene–DNA adduct as shown in Fig. 1.

After 90 days of exposure, the same organs cited above, as well as bladder, were examined for DNA adducts. Again, all organs from the low- and medium-exposure groups had levels of DNA adducts which were not visually or statistically different from those of sham controls. Doubling the amount of DNA used for TLC development to 8 μg did not produce any exposure-related adducts (i.e., a DRZ) in the low- and medium-exposure groups (data not shown). Lung, heart, and larynx DNA from the high-exposure group exhibited elevated levels of DNA adducts with DRZ and a few discrete spots. Some of the discrete spots appeared in all exposure and sham groups. The RAL values of total adducts were higher than those in the same organs at 28 days. The liver and bladder DNA, however, remained negative even at the high exposure. Representative adduct maps of lung, heart, and larynx tissues from 90-day necropsies are shown in Fig. 2. The scanning time for these samples was 8 hr. Increasing scanning times to as long as 30 hr did not reveal any additional adducts (data not shown). In addition to the P1 nuclease method for adduct enrichment, butanol extraction was also used for both lung and heart DNA from the 90-day samples. The results (data not shown) were similar. The butanol method did not reveal any adduct spots or

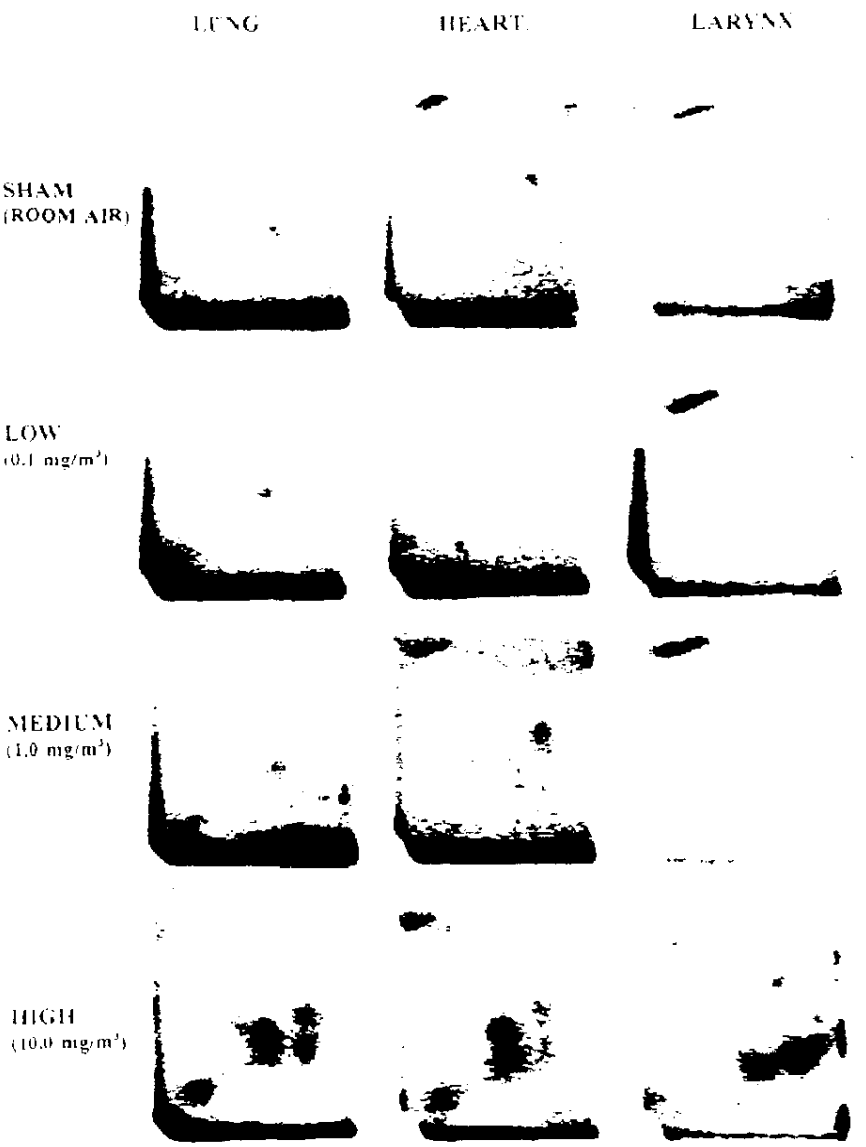

FIG. 2. DNA adduct maps of lung, heart, and larynx tissues of Sprague–Dawley rats exposed to aged and diluted sidestream smoke for 13 weeks. DNA adducts were analyzed using a P1 nuclease version of the ^{32}P-postlabeling assay. 4 μg of DNA were applied on PEI-cellulose TLC for adduct development. TLCs were scanned for 8 hr with AMBIS radioanalytic imaging system.

DRZs which were absent in the nuclease P1 method. Several TLC plates were also scanned using a PhosphorImager instrument (Molecular Dynamics, Sunnyvale, CA) for up to 16 hr. The images developed (not shown) were identical to those obtained using the Ambis after 8 hr of scanning.

The absence of discernible DRZs and statistically nonsignificant levels of total adduct level in the medium- and low-exposure animals could be due to a limit in sensitivity of the ^{32}P-postlabeling assay. To test this possibility, 90-day lung DNA samples from the high-exposure group were postlabeled and an aliquot representing 0.4 μg of DNA was spotted on TLC for adduct resolution. As shown in Fig. 3, the adduct maps of the high-exposure group obtained with 0.4 μg of DNA clearly demonstrated the presence of DRZs and other discrete spots. These results coupled with the absence of DRZs in the 4- and 8-μg samples of DNA from the medium-exposure animals effectively rule out the possibility that the absence of DRZs in the medium-exposure ani-

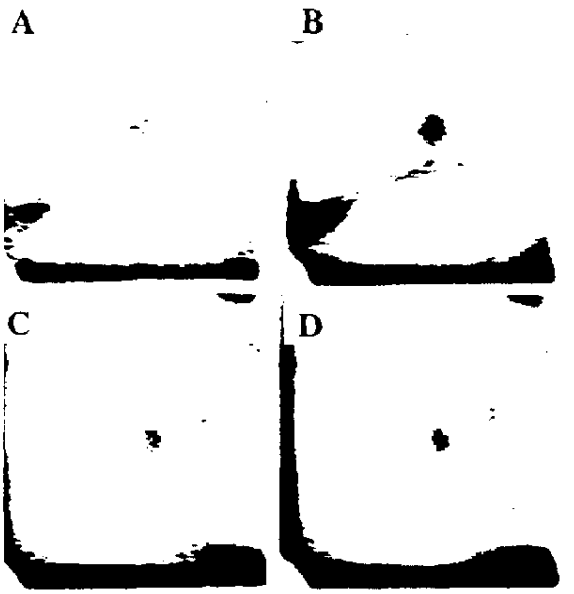

FIG. 3. DNA adduct maps of lung tissues from the high- (A, B) and the medium-(C, D) exposure groups as analyzed with 0.4 and 4 μg of DNA samples, respectively, on TLC. A PI nuclease version of ^{32}P-postlabeling assay was employed. The TLCs were scanned for 8 (A, C) and 60 hr (B, D) with AMBIS. Note the presence of DRZs in the high-exposure group (A, B), but no corresponding DRZs in the medium-exposure group.

mals is due to the lack of sensitivity of the assay. These data clearly indicate that the dose–response for DNA adduct formation is nonlinear at ADSS concentrations between 1.0 and 10 mg/m^3.

Adduct assays from animals examined 90 days after cessation of ADSS exposure exhibited significant decreases in total adduct levels compared to those before the cessation in the high-exposure group. The RAL values of all the tissue samples (lung, heart, larynx, liver, and bladder) at 28-, 90-, and 180-day necropsies are presented in Fig. 4. It is clear from these results that DNA adducts in low- and medium-exposure groups did not increase with increased duration of exposure; the adducts in lung, heart, and larynx DNA of the high-exposure group exhibited the highest RAL values after 90 days of exposure and then decreased after cessation of ADSS exposure.

The results of the chromosomal aberration assay in PAM after 28 and 90 days of exposure are presented in Table 1. Frequencies of cells with aberrations in all three ADSS-exposed groups are minimal and not different from those of the sham control in both 28- and 90-day samples. Aberrations were mostly chromatid-type rather than chromosome-type in all samples. Animals injected with the positive control, cyclophosphamide, exhibited a statistically significant increase in chromosomally aberrant cells.

DISCUSSION

In the present study, ADSS was used as a surrogate for ETS and animals were exposed subchronically to three concentrations (0.1, 1.0, and 10 mg total particulate matter (TPM)/m^3) of ADSS, representing ambient, 10-fold, and 100-fold exaggerated concentrations of the particulate matter normally found in indoor environments where smoking is occurring (Guerin *et al.*, 1992). When the fact that tobacco smoke accounts for less than 50% of RSP in indoor air (Guerin *et al.*, 1992) is considered, the ADSS concentrations in our study in reality represent 2-, 20-, and 200-fold exaggerations of field values. To evaluate the genotoxic effects of exposure, we chose two sensitive and relevant biomarkers of genotoxicity, chromosomal aberrations in pulmonary alveolar macrophages and DNA adducts in major internal organs as measured by the ^{32}P-postlabeling assay.

The ^{32}P-postlabeling assay has the advantage of high sensitivity (1 adduct in 10^{9-10} nucleotides) as well as not requiring knowledge of the chemical identity of the adducts. Therefore, it is suitable for the study of molecular dosimetry of covalent DNA binding at low concentrations of complex mixtures such as ETS. A dose–response relationship between the DNA adduct levels in the target tissues and application doses of a series of complex mixtures has been reported (Gallagher *et al.*, 1990; Jahnke *et al.*, 1990). The presence of DNA adducts in smokers has been reported, with significantly reduced levels in exsmokers and nonsmokers (Phillips *et al.*, 1988; Randerath *et al.*, 1989; Cuzick *et al.*, 1990; Phillips *et al.*, 1990; Geneste *et al.*, 1991). In rodents, adduct formation has been reported in lungs and other respiratory tissues (Bond *et al.*, 1989; Gupta *et al.*, 1989; Gairola and Gupta, 1991; Reddy and Randerath, 1990) following cigarette smoke exposure.

The discrete DNA adducts detected in the low- and medium-exposure groups were qualitatively and quantitatively similar to those observed in the sham group. Although the identity of these discrete adduct spots seen in all the tissue samples is unknown, they are also present in the high-exposure group. The adduct maps of tissues after exposure to cigarette smoke or other complex mixtures have been shown to include DRZs in addition to these discrete spots. Because of its unique appearance, a DRZ may serve as a "fingerprint" for exposure to cigarette smoke. Clearly visible DRZs were observed in select organs (lung, heart, and larynx) of the high-exposure group only. The discrete spots also increased in their intensity in the high-exposure group, indicating that exposure to ADSS at above 10 mg TPM/m^3 accelerated the preexisting DNA lesions in the rat tissues. Such observations were reported in other smoke exposure studies (Gupta *et al.*, 1989; Gairola and Gupta, 1991). Statistical analysis of the RAL values confirmed that there were more total DNA adducts present in several organs of the high-exposure group compared to those in the

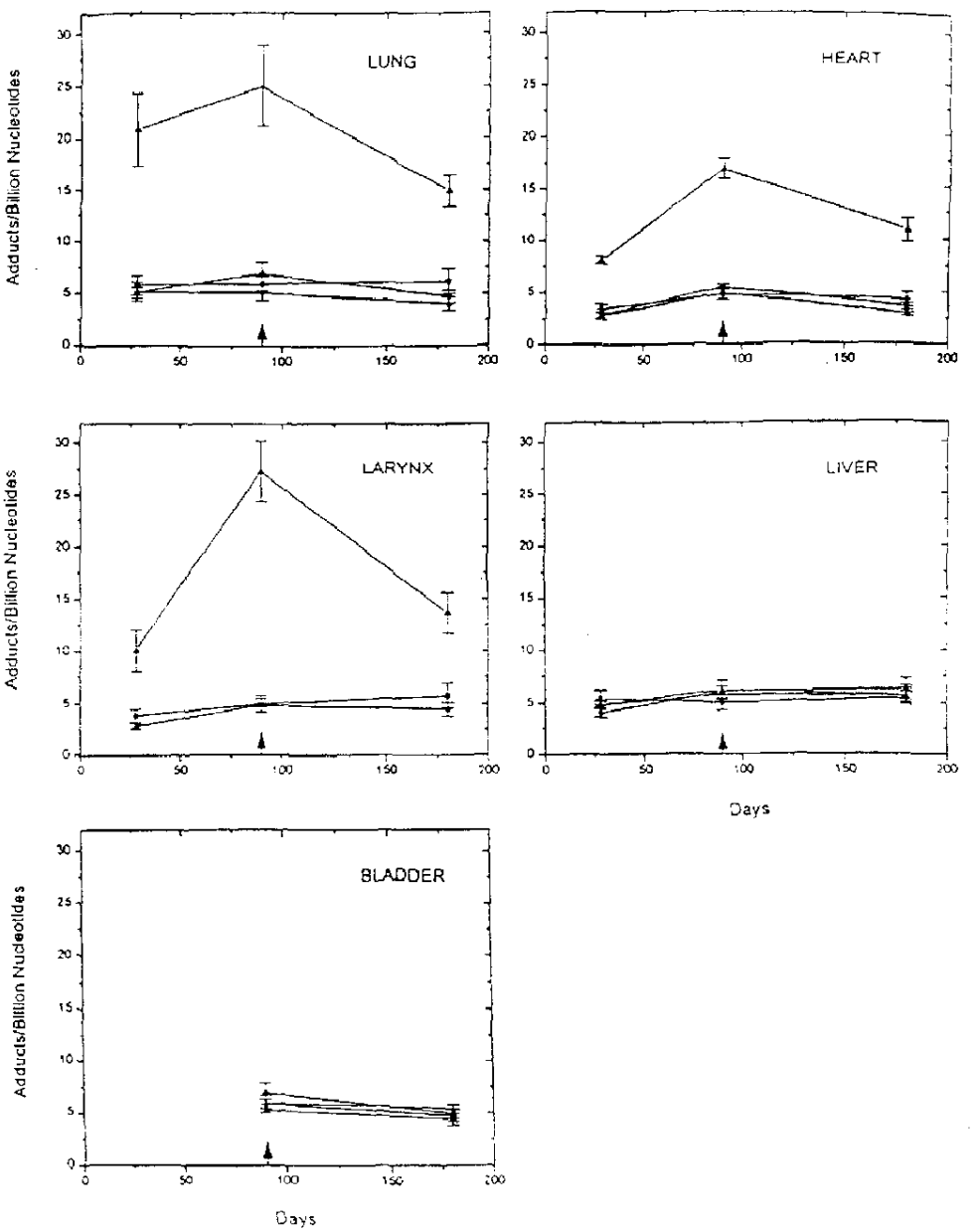

FIG. 4. Time course of adduct formation in the indicated tissues of Sprague–Dawley rats exposed to aged and diluted sidestream smoke. Adducts were determined after 28 (except bladder) and 90 days of ADSS exposure and 90 days after cessation of exposure (180 days). Arrows in the figures indicate 90 days, when the ADSS exposure ended. DNA adducts were analyzed by the P1 nuclease version of ^{32}P-postlabeling assay. Mean RAL values were determined from five animals in each exposure group. The error bars indicate SEM. —△— high; —■— medium, —♦— low; —●— sham.

sham and low- and medium-exposure groups. These results were confirmed using both P1 nuclease and butanol extraction methods for adduct enrichment. Bladder and liver did not show ADSS-related adducts even in the high-exposure group. Sprague–Dawley rats exposed to high concentrations of mainstream cigarette smoke for 32 weeks exhibited DNA adducts in lung, heart, trachea, and larynx, but not in bladder and liver (Gairola and Gupta, 1991), qualitatively matching our results with ADSS.

In studies of both humans and laboratory animals, DNA

TABLE 1

Chromosome Aberration in Pulmonary Alveolar Macrophages of Rats Exposed to Aged and Diluted Sidestream Smoke for 28 or 90 Days

Exposure groups	Number of animals	Number of cells analyzed	Percentage cells with aberration
28 days			
Sham (room air)	5	250	3.2
Low (0.1 mg/m^3)	5	250	2.8
Medium (1.0 mg/m^3)	5	250	2.0
High (10.0 mg/m^3)	5	250	4.0
90 days			
Sham (room air)	5	250	1.2
Low (0.1 mg/m^3)	4[a]	172	0.6
Medium (1.0 mg/m^3)	5	250	0.8
High (10.0 mg/m^3)	5	227	1.8
CP[b] (10 mg/kg)	2	89	13.5[c]
Saline (0.5 mg/kg)	3	150	0.7

[a] Cell preparation from one animal was lost.

[b] CP, cyclophosphamide (positive control).

[c] Significantly greater ($p < 0.05$) than sham control by Fisher's exact test.

adducts have been reported to decrease and eventually disappear upon cessation of exposure to genotoxic agents. DNA adducts in rats exposed to diesel exhaust were reduced 4 weeks after the cessation of exposure (Bond *et al.*, 1990). Lung DNA adducts from cigarette smoke-exposed rats were significantly reduced 19 weeks after cessation of exposure (Gupta *et al.*, 1989). In the present study, similar results were observed in that there was a significant decrease in RAL values in the target tissues 90 days after the cessation of exposure.

Most *in vitro* studies with concentrated cigarette smoke condensate have reported positive results in chromosome aberrations and sister chromatid exchange assays. *In vivo* studies with rodents have frequently employed bone marrow cells or lymphocytes to evaluate the clastogenic effects of cigarette smoke exposure by inhalation. In these studies, the evidence of clastogenicity of cigarette smoke exposure in these cell types has not been conclusive. Both positive (Balansky *et al.*, 1987, 1988; Putman *et al.*, 1985; Benedict *et al.*, 1984) and negative (Coggins *et al.*, 1990; Lee *et al.*, 1990; Basler, 1982; Korte *et al.*, 1981) results were reported. Recently, Rithidech *et al.*, (1989) exposed rats to approximately 100 to 200 mg TPM/m^3 for 6 hr/day, 5 days/week for 22 to 24 days and reported a significant increase in the frequencies of chromosomal aberration in the pulmonary alveolar macrophages of exposed animals. It appears that these cells, which are collected in the respiratory tract, are more sensitive to cigarette smoke or airborne chemicals because they represent direct target tissues in inhalation. The negative results in our study indicate the concentrations of ADSS tested were insufficient to induce chromosomal damage in these cells.

Determination of the shape of the dose–response curve in the low-dose region is a difficult and often unresolved task in toxicology and risk assessment. It has often been estimated by linear extrapolation of the effects from high-dose animals (Lutz, 1990). In the previous 14-day ADSS study (Lee *et al.*, 1992), we observed faint but unmistakable DRZs in the lung DNA of the high-exposure group (10 mg TPM/m^3) after 7 consecutive days of exposure. The animals in the current study were exposed 5 days a week for 13 weeks for a net total exposure of 65 days. If the product of concentration (C) times exposure time (T) is linear, one would expect to see DRZs in the medium-dose animals after 65 days since the $C \times T$ for the two studies are similar (7 days × 10 mg/m^3 = 70 mg days/m^3, 65 days × 1 mg/m^3 = 65 mg days/m^3). In the present study, the medium-exposure group (1 mg TPM/m^3) after 90 days (65 exposure days) still did not show DRZs and no RAL values higher than those of the sham group, indicating that a linear $C \times T$ extrapolation from the high-exposure 7-day study does not fit the experimental data. In addition, the clear presence of DRZs in 0.4 μg of lung DNA from the high-exposure group (Fig. 3) combined with the absence of similar adducts in 4 (Figs. 2 and 3) and 8 μg of lung DNA from the medium-exposure group provide a strong indication that the adducts in the medium-exposure group, if they exist, are less than one-tenth or one-twentieth of those formed in the high-exposure group. These data clearly support a nonlinear dose–response of DNA adduct formation by ADSS exposures, and indicate a NOEL of at least 1.0 mg/m^3.

The formation of DNA adducts in target tissue is widely regarded as a necessary initial event in the multistage process of chemical carcinogenesis. A nonlinear dose–response in DNA adduct formation with a NOEL could result from a number of host factors such as clearance of inhaled toxicants, detoxification, blocking by nucleophiles, and DNA repair. It is significant that ADSS concentrations (0.1 and 1.0 mg TPM/m^3) which represent above average levels of ETS in most indoor environments where smoking is allowed and a 10- and 20-fold increase in concentration, respectively, did not result in any exposure-related adducts after 90 days of inhalation exposure. None of the concentrations tested significantly increased chromosome aberrations in PAM. Thus, under the conditions of these studies, ADSS at 1.0 mg TPM/m^3 represents a NOEL for DNA adduct formation in lung, heart, and larynx and a NOEL of at least 10 mg/m^3 exists for the induction of chromosome aberrations in alveolar macrophages.

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